glycols) or poly(ethylene oxides), all commonly known as PEGs. One example of branched PEG is the subject of U.S. Patent 5,643,575.

In one embodiment of the invention, the average number of lysines inserted per uricase subunit is between 1 and 10. In a preferred embodiment, the number of additional lysines per uricase subunit is between 2 and 8. It being understood that the number of additional lysines should not be so many as to be a detriment to the catalytic activity of the uricase. The PEG molecules of the conjugate are preferably conjugated through lysines of the uricase protein, more preferably, through a non-naturally occurring lysine or lysines which have been introduced into the portion of a designed protein which does not naturally contain a lysine at that position.

The present invention provides a method of increasing the available non-deleterious PEG attachment sites to a uricase protein wherein a native uricase protein is mutated in such a manner so as to introduce at least one lysine residue therein. Preferably, this method includes replacement of arginines with lysines.

PEG-uricase conjugates utilizing the present invention are useful for lowering the levels (i.e., reducing the amount) of uric acid in the blood and/or urine of mammals, preferably humans, and can thus be used for treatment of elevated uric acid levels associated with conditions including gout, tophi, renal insufficiency, organ transplantation and malignant disease.

PEG-uricase conjugates may be introduced into a mammal having excessive uric acid levels by any of a number of routes, including oral, by enema or suppository, intravenous, subcutaneous, intradermal, intramuscular and intraperitoneal routes. Patton, JS, et al., (1992) Adv Drug Delivery Rev 8:179-228.

The effective dose of PEG-uricase will depend on the level of uric acid and the size of the individual. In one embodiment of this aspect of the invention, PEG-uricase is administered in a pharmaceutically acceptable excipient or diluent in an amount ranging from 10 μg to about 1 g. In a preferred embodiment, the amount administered is between about 100 μg and 500 mg. More preferably, the conjugated uricase is administered in an amount between 1 mg and 100 mg, such as, for example, 5 mg, 20 mg, or 50 mg. Masses given for dosage amounts of the embodiments refer to the amount of protein in the conjugate.

Pharmaceutical formulations containing PEG-uricase can be prepared by conventional techniques, *e.g.*, as described in Remington's Pharmaceutical Sciences, (1985) Easton, PA: Mack Publishing Co. Suitable excipients for the preparation of injectable solutions include, for example, phosphate buffered saline, lactated Ringer's solution, water, polyols and glycerol. Pharmaceutical compositions for parenteral injection comprise pharmaceutically acceptable sterile aqueous or non-aqueous liquids, dispersions, suspensions, or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. These formulations can contain additional components, such as, for example, preservatives, solubilizers, stabilizers, wetting agents, emulsifiers, buffers, antioxidants and diluents.

PEG-uricase may also be provided as controlled release compositions for implantation into an individual to continually control elevated uric acid levels in blood and urine. For example, polylactic acid, polyglycolic acid, regenerated collagen, poly-L-lysine, sodium alginate, gellan gum, chitosan, agarose, multilamellar liposomes and many other conventional depot formulations comprise bioerodible or biodegradable materials that can be formulated with biologically active compositions. These materials, when implanted or injected, gradually break down and release the active material to the surrounding tissue. For example, one method of encapsulating PEG-uricase comprises the method disclosed in U.S. Patent No. 5,653,974, which is hereby incorporated by reference. The use of bioerodible, biodegradable and other depot formulations is expressly contemplated in the present invention. The use of infusion pumps and matrix entrapment systems for delivery of PEG-uricase is also within the scope of the present invention. PEG-uricase may also advantageously be enclosed in micelles or liposomes. Liposome encapsulation technology is well known in the art. See, e.g., Lasic, D, et al., (Eds.) (1995) Stealth Liposomes, Boca Raton, FL: CRC Press.

The PEG-uricase pharmaceutical compositions described herein will decrease the need for hemodialysis in patients at high risk of urate-induced renal failure, *e.g.*, organ transplant recipients (see Venkataseshan, VS, et al., (1990) Nephron 56:317-321) and patients with some malignant diseases. In patients with large accumulations of crystalline urate (tophi), such pharmaceutical compositions will improve the quality of life more rapidly than currently available treatments.

The following examples, which are not to be construed as limiting the invention in any way, illustrate the various aspects disclosed above.

EXAMPLE 1

A. Construction of PBC, PKS and related uricase cDNAs.

Standard methods, and where applicable instructions supplied by the manufacturers of reagents, were used for preparing total cellular RNA, for PCR amplification (U.S. Patent Nos. 4,683,195 and 4,683,202, 4,965,188 & 5,075,216) of urate oxidase cDNAs, and for cloning and sequencing of these cDNAs (Erlich 1989; Sambrook et al. 1989; Ausubel 1998). PCR primers for pig and baboon urate oxidases (Table 1) were designed based on published coding sequences (Wu et al. 1989) and using the PRIME software program (Genetics Computer Group, Inc.).

Table 1. Primers for PCR Amplification of Urate Oxidase cDNA

Pig liver uricase cDNA:
sense: 5' gcgcgaattccATGGCTCATTACCGTAATGACTACA 3'.
Antisense: 5' gcgctctagaagcttccatggTCACAGCCTTGAAGTCAGC 3'.
D3H baboon liver uricase cDNA:
sense: 5' gcgcgaattccATGGCCCACTACCATAACAACTAT 3'
antisense: 5' gcgcccatggtctagaTCACAGTCTTGAAGACAACTTCCT

Restriction enzyme sequences (lowercase) introduced at the ends of the primers are sense (pig and baboon) EcoRI and NcoI; antisense (pig) NcoI, HindIII, XbaI; antisense (baboon) NcoI. In the case of baboon sense primer, the third codon GAC (Aspartate) present in baboon urate oxidase (Wu et al. 1992) was replaced with CAC (Histidine), the codon that is present at this position in the coding sequence of the human urate oxidase pseudogene (Wu et al. 1992). For this reason the recombinant